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Relationship Between Modulation of
Glomus Cell K^+ Current and Hypoxia Transduction
in the Rat Carotid Body

Phillip Ming-Da Cheng

Yale University

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and Hypoxia Transduction in the Rat Carotid Body

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Phillip Ming-Da Cheng

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RELATIONSHIP BETWEEN MODULATION OF GLOMUS CELL K^+ CURRENT AND HYPOXIA TRANSDUCTION IN RAT CAROTID BODY. Phillip M. Cheng and David F. Donnelly. Section of Respiratory Medicine, Department of Pediatrics, Yale University School of Medicine, New Haven, CT.

This study was undertaken to assess a hypothesis of rat carotid body chemotransduction, namely that the increase in sinus nerve activity in response to hypoxia is mediated by an inhibition of glomus cell K^+ current. Sinus nerve activity and glomus cell membrane currents were measured simultaneously in intact rat carotid bodies, *in vitro*. Brief hypoxia (30-45 s) caused a rapid and marked increase in nerve activity to 21.6 ± 2.7 times baseline spiking frequency ($n=59$), but caused no significant change in glomus cell membrane resistance, outward current, or holding current. Tetraethylammonium (TEA, 20 mM) reduced glomus cell outward current to $55 \pm 7\%$ ($n=15$) of predrug current, but nerve activity increased only slightly to 2.0 ± 0.3 times baseline spiking frequency. Brief hypoxia in the presence of TEA caused a marked increase in nerve activity to 30 ± 13 times baseline spiking frequency ($n=3$). Charybdotoxin (10 nM, $n=11$) and apamin (100 nM, $n=13$) reduced outward current by $12.1 \pm 3.0\%$ and $11.4 \pm 6.1\%$ respectively, but did not significantly affect nerve activity, glomus cell membrane resistance, or holding current. The results suggest that hypoxia inhibition of glomus cell K^+ current is not the primary initiating step in the nerve response to hypoxia in the rat carotid body.

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It is a pleasure for me to acknowledge Dr. David F. Donnelly for his guidance, teaching, patience, generosity, and friendship over the past three years. His dedication to the vexing puzzle of signal transduction in the carotid body is inspiring, and his singular ability to generate a constant stream of interesting theoretical and practical questions helped maintain an intellectually challenging environment in which to think and work. I can only hope that he derived as much pleasure and excitement as I did from our brief collaboration.

AUTHOR'S NOTE

The experimental results described herein have been published in the *Journal of Neurophysiology* (Cheng and Donnelly 1995).

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INTRODUCTION

Clinical Physiology of the Carotid Body

The carotid bodies are paired organs located at the bifurcations of the common carotid arteries. The first anatomical description of the carotid body is attributed to Winslow in 1732 (Eyzaguirre and Zapata 1984), but the detailed anatomical structure of the organ was not well characterized until De Castro's pioneering microscopic studies were published in 1928. De Castro, regarded by some as the founder of chemoreceptor science (O'Regan and Nolan 1994), deduced from the anatomical relationships he observed that the carotid body served a "tasting" function for substances transported in the blood. His insightful conjecture foreshadowed Heymans' Nobel-prizewinning physiological experiments in the 1930's, which demonstrated the importance of chemoreception within the dog carotid sinus in the control of respiration (Breathnach 1994).

Each carotid body is about 1 mm³ in humans, innervated both by the carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve (cranial nerve IX), and by a sympathetic nerve branch from the superior cervical ganglion. The carotid body is well-vascularized, with one of the highest ratios of blood flow to organ weight in the body: approximately 2000 ml/min/100g tissue (Taylor et al. 1989) compared to 60 ml/min/100g in the brain and 64-151

ml/min/100g in the left ventricle (Fidone and Gonzalez 1986). Such a high perfusion-to-size ratio results in a very small arterial-venous O_2 difference despite a high metabolic rate. As a result, the carotid body is sometimes described as responding to levels of essentially arterial PO_2 , PCO_2 , and pH (Taylor et al. 1989; West 1995).

The actual physiology appears to be more complicated, since multiple studies have documented significant discrepancies between the carotid body tissue PO_2 and the venous PO_2 . Microelectrode measurements *in vivo* of cat carotid body tissue PO_2 under normoxic conditions, for instance, have yielded mean PO_2 values around 20 mmHg (Acker et al. 1971; Acker 1989). On the other hand, another group using similar techniques has reported significantly higher tissue PO_2 's, in the range of 40-100 mmHg (Whalen et al. 1981). Although differences between venous and tissue PO_2 have been attributed to significant arteriovenous shunting (Acker and O'Regan 1981), both the precise degree and the physiologic significance of these differences remain topics fraught with controversy (Delpiano and Acker 1980; Whalen et al. 1981; Degner and Acker 1986; Rumsey et al. 1991).

In any event, on a macroscopic level, arterial hypoxia, hypercapnia, and acidosis each cause an increase in the sensory discharge frequency of the afferent sinus nerve fibers, which in turn stimulates the medullary respiratory center to cause an increase in ventilation. The sinus nerve

response is graded, exhibiting an increased discharge frequency with worsening hypoxia, hypercapnia, or acidosis. The carotid body thus serves to adjust respiratory reflexes to maintain physiologically acceptable levels of arterial PO_2 , PCO_2 , and pH (Fidone and Gonzalez 1986).

Of these chemosensory functions, the response to hypoxia seems to be the most important physiologically. The carotid bodies appear to be the only organs in humans to directly stimulate ventilation in response to hypoxemia, in contrast to dogs and cats, in which aortic bodies also contribute to hypoxic ventilatory responsiveness (Whipp 1994). A study of asthmatic patients with bilateral carotid body resections demonstrated that these patients exhibit no increase in tidal volume nor respiratory rate in response to acute hypoxemia (Lugliani et al. 1971). Sensitivity of the carotid body to changes in arterial PO_2 begins around 500 mmHg (West 1995). However, the relation between sinus nerve firing rate and arterial PO_2 is highly nonlinear, with a minimally detectable nerve response until arterial PO_2 drops below 100 mmHg (West 1995). An actual increase in ventilatory response is typically not seen until the P_aO_2 drops below 60 mmHg (Staub 1991). Hypercapnia and acidosis can markedly potentiate the nerve response of the carotid body, however, causing firing rates up to 20% of the maximum at normal oxygen tensions (Taylor et al. 1989).

In humans, the physiologic importance of the carotid

body has long been a subject of controversy. Within the past 50 years, therapeutic carotid body resection (glomectomy) has been performed in several clinical settings under the assumption that the carotid body is not essential for maintaining blood gas and pH homeostasis in humans. Nakayama in the 1940's and 1950's performed primarily unilateral and some bilateral glomectomies in Japan to treat asthmatics. In his study of 3,914 patients, 81.3% of patients expressed subjective "cure or improvement" after six months, with 58% maintaining good results after 5 years (Nakayama 1961). He further noted that results of bilateral glomectomy were comparable with those of unilateral glomectomy (Nakayama 1961). The physiologic rationale for this operation was never clear, however, (Barnes 1994) and subsequent controlled studies showed no clear benefit from unilateral glomectomy (O'Rourke and O'Rourke 1964; Marschke et al. 1965; Curran et al. 1966). In fact, a surgical placebo effect could not be completely excluded, as demonstrated by the case of one of the patients of Marschke et al. (Marschke et al. 1965) who was in the control group receiving a sham unilateral carotid body operation; she subsequently reported being able to lie flat without dyspnea for the first time in years and was astonished to find that she was in the control group (Stulbarg and Winn 1989).

More recently, in the 1960's and 1970's, Winter performed bilateral glomectomies to relieve dyspnea in

patients with COPD (Winter 1973), under the theory that removal of the carotid bodies leads to a reduction of bronchospasm. The original basis for this reasoning was a report that stimulation of the carotid bodies in the dog leads to airway constriction, suggesting a role for the carotid body in a reflex bronchomotor loop (Nadel and Widdicombe 1962). Most of Winter's glomectomized patients did in fact report significant relief of dyspnea, although subsequent studies suggested that this was due to the loss of the hyperpneic response to hypoxia rather than to effects on bronchoconstriction (Stulbarg and Winn 1989). However, no controlled study of the efficacy and dangers of this operation was ever performed, and because of the lack of data and widespread suspicion that the procedure was dangerous on physiologic grounds, Medicare reimbursement for the procedure was withdrawn (Stulbarg and Winn 1989).

Yet because the incidence of death from CO₂ narcosis and sleep apnea was surprisingly low in the patients with COPD who underwent glomectomy (Severinghaus 1989), the question remains as to the physiologic importance of hypoxia chemosensitivity. Significantly, however, it has long been noted that insensitivity to hypoxia among older native high-altitude dwellers leads to secondary polycythemia, pulmonary hypertension, and right heart failure (Severinghaus 1976). In addition, there have been anecdotal reports of critical reductions in arterial oxygenation in glomectomized patients

exposed to a hypoxic stress. Nakayama's procedures were terminated after two Japanese athletes died post-glomectomy while attempting long underwater swims (Severinghaus 1989). Chang reported a case of a boy who, after glomectomy, experienced repeated episodes of loss of consciousness, possibly related to his absent ventilatory response to hypoxia (Chang et al. 1978).

More recently, a study of asthmatics with a history of near-fatal asthma attacks (i.e., requiring mechanical ventilation or resulting in unconsciousness) showed that these patients had a significantly reduced ventilatory response to hypoxia compared to asthmatic controls without a history of near-fatal attacks (Kikuchi et al. 1994). Compared to controls, these patients also exhibited a significantly reduced perception of dyspnea, as measured by having the patients breathe through a series of tubes of increasing resistance. Furthermore, the perception of dyspnea measured in this manner correlated positively to hypoxic, but not hypercapnic, chemosensitivity in all groups studied.

Though the precise physiologic relationship between dyspneic perception and arterial chemoreception requires further elucidation, these results nevertheless suggest that ventilatory responsiveness to hypoxia, though not critical to ventilatory regulation in normal humans, may be an important contributor to morbidity and mortality in patients

with underlying pulmonary disease. Indeed, the issue of the possible beneficial effects of glomectomy in patients with obstructive pulmonary disease may involve a trade-off between dyspnea and pulmonary insufficiency, as noted in an editorial by Wasserman (Wasserman 1978).

It has recently been proposed that a similar trade-off occurs in patients with heart failure. Chugh and colleagues cite observations that the arterial hypoxic chemoreflex is upregulated in heart failure patients, thereby accounting for the increase in ventilatory drive with exercise seen in such patients (Chugh et al. 1996). Though an enhanced chemoreceptor reflex might confer some protection against tissue hypoxia, it could also contribute to the dyspnea seen in patients with chronic heart failure. Further examination of the nature of such trade-offs, and of the possibility and indications for pharmacologic targeting of the chemoreceptor reflex, appears warranted.

Cellular and Molecular Physiology of the Carotid Body

The carotid body is anatomically complex (Fig. 1), consisting of glomus (type I) and sustentacular (type II) cells. The glomus cells are spherical, derived from the neural crest, and exhibit a heterogeneous population of cytosolic granules. The sustentacular cells are glial in nature and lack specialized organelles. Branches of the carotid sinus nerve innervate the glomus cells, and

processes of the sustentacular cells encapsulate clusters of glomus cells (Fidone and Gonzalez 1986; Peers and Buckler 1995).

The site of chemoreception in the carotid body has been the subject of considerable controversy until relatively recently. Although de Castro originally proposed that the glomus cells were the chemosensors, over the past several decades each of the components of the glomerular complex (glomus cell, sustentacular cell, and nerve ending) has been proposed as the transducer element (Fidone and Gonzalez 1986). It is now generally accepted that chemoreception within the carotid body occurs in the glomus cells. Preliminary evidence for this theory included experiments in which the rabbit carotid body was cryocoagulated, after which the carotid sinus nerve fibers were allowed to regenerate into the carotid bifurcation. It was discovered that the physiological response of a given preparation was related to the number of glomus cells remaining (Verna et al. 1975; Leitner et al. 1981).

Further evidence for glomus cells as the primary chemosensory element was obtained from work focusing on catecholamine biochemistry and physiology in the carotid body. Numerous studies in the 1970's demonstrated that glomus cells contain the enzymatic apparatus to synthesize catecholamines, that catecholamines (primarily dopamine, with lesser amounts of norepinephrine) are stored in the

carotid body, and that catecholamine synthesis is induced by hypoxia (Fidone and Gonzalez 1986; O'Regan and Nolan 1994). It was further shown that glomus cell catecholamine secretion is closely linked temporally with changes in nerve activity (Donnelly 1993a) and that blockers of secretion inhibit the nerve response (Fidone et al. 1982; Donnelly 1993a).

The mechanism by which the glomus cells might sense hypoxia, however, remains poorly understood. Most models of chemotransduction propose a rise in intracellular calcium as a necessary step. Early studies of dopamine release in the carotid body demonstrated a requirement of extracellular calcium (Fidone et al. 1982). Subsequent microfluorimetric studies on isolated glomus cells showed that intracellular calcium concentration rises in hypoxia (Biscoe and Duchen 1990b; Biscoe and Duchen 1990c; Lopez-Barneo et al. 1993; Buckler and Vaughan-Jones 1994b; Peers and Buckler 1995). The rise in calcium concentration has been noted to be graded, increasing as PO_2 decreases in a manner paralleling the increasing afferent fiber discharge (Biscoe and Duchen 1990c; Buckler and Vaughan-Jones 1994b; Peers and Buckler 1995).

More controversial is the question of how hypoxia leads to such an increase in intracellular free calcium. Two major hypotheses have emerged (Fig. 2). Biscoe and Duchen (Biscoe and Duchen 1990a) have argued that hypoxia leads to

the rise of glomus cell $[Ca^{2+}]_i$ via calcium release from intracellular stores. This conclusion was based initially upon observations in rabbit glomus cells that the rise in $[Ca^{2+}]_i$ in isolated glomus cells during anoxia or exposure to cyanide persists, although reduced in amplitude, in a Ca^{2+} -free solution (Biscoe et al. 1989; Biscoe and Duchen 1990c). It was also noted that a rise in $[Ca^{2+}]_i$ was still detectable in the presence of externally applied inorganic and organic calcium channel blockers (Biscoe and Duchen 1990a).

Biscoe and Duchen further proposed that mitochondrial calcium stores contribute significantly to the rise in $[Ca^{2+}]_i$ (Biscoe and Duchen 1989; Biscoe et al. 1989; Biscoe and Duchen 1990c; Biscoe and Duchen 1990b). This proposal originated from their observation that the mitochondrial uncoupler FCCP (carbonyl cyanide-4-trifluoromethoxyphenyl-hydrazine) causes a rapid rise in $[Ca^{2+}]_i$ equivalent in amplitude to the response to either cyanide or anoxia (Biscoe and Duchen 1990c; Biscoe and Duchen 1990a). They subsequently demonstrated that a graded depolarization of mitochondrial membrane potential with hypoxia correlated with changes in $[Ca^{2+}]_i$ (Duchen and Biscoe 1992a; Duchen and Biscoe 1992b).

In contrast, an alternative hypothesis suggests that hypoxia modulates glomus cell plasma membrane currents. Both rabbit and rat glomus cells exhibit inward and outward

voltage-dependent conductances, with some variation between species. In the rabbit, for instance, inward conductances include an L-type voltage-gated calcium channel (Obeso et al. 1992) and a transient voltage-gated sodium conductance blocked by tetrodotoxin (TTX) (Lopez-Barneo et al. 1988). Outward conductances include both calcium-dependent and calcium-independent potassium channels. The calcium-sensitive channels include at least one high-conductance (210 pS) channel (Ganfornina and Lopez-Barneo 1992), and possibly a low-conductance channel blocked by apamin (Duchen et al. 1988). Calcium-insensitive channels include at least two relatively small-conductance channels of 40 and 16 pS (Ganfornina and Lopez-Barneo 1992).

Rat glomus cells similarly exhibit an L-type voltage-gated calcium conductance (Fieber and McCleskey 1993), but the presence of sodium channels is less clear. Various groups have reported either that rat glomus cells do not have sodium channels (Fieber and McCleskey 1993), or that sodium channels are present at low density (Stea and Nurse 1991). Like rabbit glomus cells, rat glomus cells exhibit both calcium-dependent and calcium-independent potassium conductances. The calcium-sensitive potassium channel shows high-conductance (190 pS) and is blocked by charybdotoxin, but not apamin (Peers 1990a; Wyatt and Peers 1995). A voltage-dependent calcium-independent channel sensitive to 4-aminopyridine (4-AP) has also been reported (Peers 1990b).

It was proposed that hypoxia-mediated inhibition of potassium currents, in particular, would lead to cell membrane depolarization, thereby activating voltage-gated Ca^{2+} channels and allowing an influx of calcium from the extracellular space. This hypothesis originated from a report in 1988 that hypoxia inhibits an outward K^+ current measured by whole-cell patch clamp in dispersed rabbit glomus cells, with Na^+ and Ca^{2+} currents unaffected (Lopez-Barneo et al. 1988). The K^+ current inhibition was attributed to the closing of a 40 pS, Ca^{2+} -independent, O_2 -sensitive channel which could be blocked by 4-AP (Ganfornina and Lopez-Barneo 1992; Peers and Buckler 1995). The rabbit glomus cells, which can generate repetitive action potentials, were further shown to exhibit an increase in their spontaneous discharge frequency during hypoxia (Lopez-Barneo et al. 1993), a phenomenon attributed to hypoxia-mediated cell membrane depolarization (Peers and Buckler 1995).

Subsequently, these observations were extended to the rat carotid body (Peers 1990c). Notably, in contrast to rabbit glomus cells, rat glomus cells are unexcitable and simply undergo membrane depolarization in response to K^+ current inhibition (Donnelly 1993b). Moreover, the inhibited K^+ current in rat glomus cells appears to be of a calcium-dependent variety, in contrast to the Ca^{2+} -independent O_2 -sensitive channel in rabbit glomus cells

(Peers 1990c). Nevertheless, further support was lent to the membrane current model by experiments demonstrating that agents which cause sinus nerve excitation also caused an inhibition of glomus cell outward current in the rat. These agents include cyanide (Peers and O'Donnell 1990; Donnelly 1993b), doxapram (Peers 1991), and acidity (Peers 1990a).

Despite its popularity, the membrane current model for hypoxia transduction in the carotid body has remained controversial. Studies which attempted to determine single K^+ -channel open probability as a function of PO_2 showed that channel inhibition was most significant over a PO_2 range of 150 to 120 mmHg. Curiously, this is a PO_2 range higher than that which normally excites the carotid body (Ganfornina and Lopez-Barneo 1991; Peers and Buckler 1995). Furthermore, interestingly, Biscoe and Duchen have reported in their preparation of rabbit glomus cells an *increase* in potassium current, as well as glomus cell hyperpolarization, during exposure to cyanide (Biscoe and Duchen 1989). Because this observation seemingly directly contradicts other reports regarding glomus cell membrane current responses to cyanide (Peers and O'Donnell 1990; Donnelly 1993b), a question arises as to whether the laboratory methods used for dissociation and culture of glomus cells can cause changes in the biophysical properties of these cells (Donnelly 1995).

In order to avoid the problem of artefactual alteration

of glomus cell physiology by chemical dissociation, we have carried out a series of experiments in a preparation of the intact mature rat carotid body. The carotid body is arranged so that glomus cell membrane currents as well as sinus nerve activity can be measured simultaneously in the intact organ. Our goal has been to test the membrane current model of hypoxia transduction by examining *in vitro* the response of the sinus nerve to modulation of glomus cell membrane currents by potassium channel blockers.

Previous experiments in this laboratory demonstrated that in rat carotid bodies, the potassium channel blocker 4-AP causes neither glomus cell catecholamine release nor sinus nerve excitation to the same degree that severe hypoxia does (Doyle and Donnelly 1994). In the experiments described herein, we develop and extend these observations using a variety of potassium channel blockers of varying specificity for potassium channel subtypes, while in addition directly measuring glomus cell membrane currents via a perforated-patch technique (Horn and Marty 1988; Rae et al. 1991). Amphotericin, a cation-channel forming agent used clinically as an antifungal, was used in the patch pipette to gain access to cell membrane voltages and currents while minimizing disturbances to the glomus cell's intracellular chemical milieu during our measurements. Furthermore, the recordings were made during superfusion of the carotid body with saline buffered by bicarbonate instead

of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), which has been used previously in this laboratory (Donnelly 1995). This buffer substitution may be of significance, since prior observations by other laboratories have suggested that an absence of bicarbonate may severely impair the hypoxia response in the carotid body (Shirahata and Fitzgerald 1991).

Our general hypothesis was that the sinus nerve response to hypoxia is mediated by inhibition of glomus cell K^+ current and therefore 1) hypoxia-induced K^+ current inhibition should occur before the nerve response to hypoxia, and 2) K^+ channel blockers should emulate severe hypoxia.

METHODS

Note. All carotid body dissections were performed by D.F. Donnelly. All subsequent procedures listed were performed by either P.M. Cheng or D.F. Donnelly. The majority of experimental runs were performed by P.M. Cheng.

Organ isolation and cell identification. Carotid bodies were isolated from 32 rats, aged 17 ± 3 days (mean $\pm$ SD), at which time the respiratory (Eden and Hanson 1987) and chemoreceptor (Kholwadwala and Donnelly 1992) responses are mature or nearly mature. Rats were anesthetized by placement in a closed chamber in which the atmosphere was saturated with methoxyflurane vapor. Following deep anesthesia indicated by repetitive slow breathing and an absence of motor movements, the rats were removed from the chamber and decapitated. The region around the carotid bifurcation was rapidly removed and placed in chilled, oxygenated (95% O₂, 5% CO₂) Ringer's solution (in mM: 125 NaCl, 3 KCl, 24 NaHCO₃, 1.25 NaH₂PO₄, 1.2 CaCl₂, 1.2 MgSO₄ and 10 glucose). The carotid bodies were dissected free along with a length of the cut sinus nerve and placed in oxygenated Ringer's solution containing 0.02% collagenase (Boehringer type P) and 0.02% protease (Sigma type XIV) at 26°C for 30 min. Following exposure to the enzyme solution, the connective tissue around the carotid body and sinus nerve was carefully

removed with a sharpened pipette. After cleaning, the carotid body was placed at the bottom of a chamber mounted on an inverted microscope and superfused at 2-3 ml/min with Ringer's solution. The superfusate PO_2 next to the carotid body was measured with a platinum wire electrode, insulated in a glass pipette, and covered with a butylacetate membrane. The O_2 electrode was calibrated by perfusion of the chamber with saline equilibrated with 20% O_2 , 5% CO_2 , balance N_2 ($PO_2 \approx 150$ mmHg) and equilibrated with 5% CO_2 , balance N_2 with the addition of glucose oxidase ($10 \mu\text{g/ml}$) which served as an oxygen scavenger ($PO_2 \approx 0$ mmHg).

Chemoreceptor afferent recordings were obtained from the cut end of the sinus nerve as previously described (Donnelly and Kholwadwala 1992; Kholwadwala and Donnelly 1992).

Temperature of the superfusate was maintained at 28-30°C. This lower temperature was used because, in preliminary experiments, membrane currents were unstable over 10-15 min time when recorded at 35°C.

For most experiments the electrode was filled with fluoride-based pipette solution (in mM: 125 K-fluoride, 5 EGTA, 1.53 Ca^{+2} , 9 glucose, 24 HCO_3). A fluoride-based pipette solution has been extensively used in other laboratories for the study of glomus cells (Duchen et al. 1988; Lopez-Barneo et al. 1988) and thus facilitates comparison of experimental results. In some cases K-

aspartate was substituted for K-fluoride. The solution was bubbled with 20% O₂, 5% CO₂, balance N₂ prior to filling the electrodes. Whole cell membrane currents were generally recorded with the perforated-patch technique (Horn and Marty 1988; Rae et al. 1991) using amphotericin B as the channel forming agent (Rae et al. 1991). A fresh stock solution of amphotericin B in DMSO (60mg/ml) was added to the pipette solution to give a final concentration of 80µg/ml. This solution was used to back-fill the patch pipette after filling the tip with amphotericin-free pipette solution (Rae et al. 1991). In other experiments, conventional whole cell recordings were obtained using the same pipette solution. Prior to seal formation, the electrode was advanced into the tissue using the 'blind patch' technique under constant ejection pressure until resistance changes consistent with a cell membrane were apparent.

The experimental apparatus is diagrammed in Figure 3. Glomus cells were voltage-clamped using an Axopatch 1-D amplifier (Axon Instruments). The Axopatch was controlled by PCLAMP software (Axon) on an MS-DOS based microcomputer (Gateway 486DX2 50 MHz) for the measurement of passive cell characteristics. Software written by D.F. Donnelly and modified by P.M. Cheng in Turbo Pascal was used to define the Axopatch voltage output tracings for the measurement of active membrane currents, and to record and integrate all

membrane current, nerve spike, and PO_2 inputs. A Labmaster TL-1 A/D converter (Scientific Solutions) interfaced between the microcomputer and the various analog input devices (Axopatch, window discriminator for nerve spike activity, and O_2 electrode).

Experimental Paradigm: Three criteria were imposed for an acceptable cell recording. i) The initial seal resistance must be greater than 4 Gohms. This limit was imposed because it is approximately 3-4x greater than the cell input impedance as measured following the start of whole-cell recording. In some cases using perforated-patch access this parameter could not be measured due to the rapidity of patch perforation. ii) An outward current was evoked by a depolarization above -20mV . iii) The charging current following a step hyperpolarization must be well fit to a single exponential.

Passive Properties: Passive cell characteristics were estimated based on the response to a step hyperpolarization of 20mV from a holding potential of -60mV . The current response was fitted to a 3rd order exponential and the time and magnitude of the terms recorded. Access resistance (R_a) was estimated from the magnitude of the current step at the start of the hyperpolarization pulse. Membrane resistance (R_o) was estimated from the steady-state current response.

Somal capacitance (C_m) was estimated from the time constant of the first exponential divided by the access resistance.

Active membrane currents: The I/V profile for each cell, with and without linear leak subtraction, was elicited over the voltage range of -50 to $+60$ mV from a holding potential of -60 mV. Cells were considered as candidate glomus cells if they evidenced a voltage-activated outward current above a threshold of -20 mV. This response is consistent with that observed previously on isolated glomus cells (Peers 1990a; 1990c; Stea and Nurse 1991; Donnelly 1993b), and is different than that recorded for non-glomoid, carotid body cells (Duchen et al. 1988; Urena et al. 1989; Donnelly 1993b).

Effect of hypoxia: Data were simultaneously acquired for the afferent nerve activity, electrode current and PO_2 meter output. The output from the window discriminator was continuously counted and stored (1/sec); PO_2 meter output was also sampled (1/sec). The membrane current was recorded every 10 sec prior to and during a step hyperpolarization of 20 mV (50 msec duration) and prior to and during a depolarization to $+40$ mV (70 msec duration). Hypoxia was induced by switching the perfusate for 30-45 sec from Ringer's saline equilibrated with 20% O_2 , 5% CO_2 balance N_2 ($PO_2 \approx 150$ mmHg) to saline equilibrated with N_2 and containing

glucose oxidase (10 μ g/ml), an oxygen scavenger ($PO_2 \approx 0$ mmHg).

Effect of K⁺ channel blockade: Drugs which alter K⁺ channel activity were used to help substantiate that recordings were obtained on glomus cells and to examine the relationship between changes in cell currents and nerve activity. In some cases, verapamil (5-10 μ M) was added to the perfusate to inhibit calcium current and thereby alter calcium-dependent K⁺ currents. K⁺ currents were directly antagonized with tetraethylammonium chloride (TEA, 20mM) which replaced an equal amount of NaCl or with specific blockers of Ca-dependent K⁺ currents, apamin (100nM) or charybdotoxin (10nM). In some cases, brief anoxia (40s duration) was superimposed on the TEA blockade. Separate batches of apamin and charybdotoxin were purchased from Sigma and Research Biomedical and were dissolved as stock solutions of 10 μ M in 150mM NaCl.

Data Analyses: The transient response to a step hyperpolarization was analyzed for R_a , R_o and C_m . The cell holding current, membrane resistance and outward current were calculated every 10 sec before, during and following hypoxia or drug exposure. Outward current was measured as the current difference before and at the end of a depolarization pulse. For each experiment 4 values were calculated and recorded: the currents at the start and 1 min

into a test period; the currents at the peak of the nerve response to hypoxia or drug perfusion and the currents at two min into the washout period. Univariate ANOVA with repeated measures was used to test the null hypothesis that no change in variables occurred over time (during which hypoxia or drug treatment occurred). If the F statistic (ratio of within-group to between-group variability) was significant, specific paired t-tests were employed for post-hoc comparisons between specific time points, e.g., during hypoxia vs pre-hypoxia. Level of significance for the F statistic or t-test was set at $p < 0.05$.

RESULTS

Recordings were obtained on 136 cells in which the activation of outward voltage-dependent current was consistent with that expected of glomus cells, i.e., rapid activation of a voltage-dependent outward current above a threshold of -20mV (Fig. 4). These I/V characteristics are consistent with that previously reported for dissociated rat glomus cells (Peers 1990b; Peers and O'Donnell 1990; Stea and Nurse 1991). Previous studies of dissociated rat glomus cells demonstrated an inhibition of outward current by verapamil (Peers 1990b). Verapamil (5-10 μ M) also caused an inhibition of the outward current of the purported glomus cells of the present study. In the presence of verapamil, outward current (at +40mV) was reduced to $54.6 \pm 7.5\%$ of pre-drug current (n=8), and this inhibition reversed during the washout of the drug (Fig. 5).

Passive Properties: In approximately half of the recordings, the current response to a step hyperpolarization could be well fit to a single exponential (Fig. 4). In 52/136 cells, greater than 95% of the current was in the first exponential and analysis of these cells yielded an average somal capacitance of 7.47 ± 0.54 pF (mean $\pm$ SEM). Average membrane resistance for the entire population, was 820 ± 187 Mohms for recordings using perforated-patch access

and 1433 ± 290 Mohms for conventional whole cell recordings.

Response of nerve and glomus cell to hypoxia: Switching the perfusate PO_2 from 150 mmHg to 0 mmHg for 30-45 sec caused a large and significant increase in afferent nerve activity (Fig. 6). Peak nerve activity for all hypoxia runs was 21.6 ± 2.7 times baseline spiking frequency ($n=59$). The time point for the hypoxia response of the cell under study was determined by the peak of the nerve response (Fig. 6).

Analysis of the data obtained using perforated-patch recordings over a hypoxia exposure period showed no significant change over time. Holding current was unchanged ($F=0.22$, $p=0.9$, $n=24$ trials on 18 cells), membrane resistance was unchanged ($F=0.72$, $p=0.5$) and outward current was unchanged ($F=1.71$, $p=0.17$) (Fig. 6). Although no significant population response was observed, some individual runs showed changes consistent with our hypothesis. For instance, in the cell response pictured in Figure 8, the hypoxia-induced increase in nerve activity occurred at the same time as a reversible reduction in cell outward current. In this case, there was no apparent effect on holding current or membrane resistance. Responding cells, e.g., that shown in Figure 7, were a minority of the cell sample (Fig. 8), and only 4 of 24 cells responded to hypoxia with a substantial decrease in outward current

during hypoxia.

With conventional patch-clamp recordings, nerve activity increased by 17.0 ± 2.4 fold, but holding current was unchanged ($F=0.28$, $p=0.8$, 35 trials on 30 cells), and membrane resistance was unchanged ($F=0.07$, $p=0.9$). Outward current was significantly altered over the experimental period ($F=11.4$, $p<0.01$), and the value during hypoxia was significantly lower than during the control period ($p<0.01$). However, the outward current did not significantly increase during the reoxygenation period.

Response of nerve and glomus cell to K^+ channel blockers: If inhibition of glomus cell K^+ channels is the initial step in the hypoxia transduction cascade, then addition of K^+ channel blocking agents would be expected to increase nerve activity. TEA (20mM) altered nerve activity over the experimental period ($F=8.5$, $p<0.01$, $n=20$) due to an increase of 2.8 ± 0.8 Hz (2.0 ± 0.3 times baseline) during TEA exposure ($p<0.01$). This increase in frequency was reversed during washout ($p<0.05$).

TEA caused significant changes in membrane currents for cells recorded using the perforated-patch and conventional patch technique. In perforated-patch recordings, outward current was altered by TEA ($F=10.1$, $p<0.01$, $n=15$ runs on 10

cells), due to a decrease to $55 \pm 7\%$ of control ($p < 0.01$) (Fig. 9). Membrane resistance was significantly increased by TEA ($p < 0.01$) and holding current significantly increased in the positive direction ($p < 0.01$). A similar effect of TEA on outward current was observed for cells recorded in the conventional patch clamp mode. Outward current decreased from 380 to 214 pA during TEA exposure ($p < 0.05$). However, there was no significant change in holding current ($p = 0.2$) or membrane resistance ($p = 0.4$) during TEA exposure.

In order to test whether the nerve remained responsive to hypoxia in the presence of TEA, anoxia was superimposed on the TEA inhibition. Nerve activity rapidly increased during anoxia to 30 ± 13 times baseline ($n = 3$) and returned to baseline during reoxygenation (Fig. 10). Peak single-fiber activity was 23 Hz for the unit shown in Figure 10, which is a normal peak discharge frequency for rat carotid body of this age (Kholwadwala and Donnelly 1992).

Previous results have indicated that hypoxia (Peers 1990c) and acidity (Peers 1990a; Buckler and Vaughan-Jones 1994b) modulate calcium-dependent K^+ currents, suggesting that these currents may be particularly important for rat carotid body transduction. Accordingly, changes in cell variables and nerve activity were tested in response to specific $g_K(\text{Ca})$ blockers, charybdotoxin and apamin. For all

experiments using these blockers, cells were recorded using conventional whole-cell configuration. During apamin exposure, no significant changes in membrane resistance ($F=0.25$, $p=0.86$, $n=13$ runs on 13 cells), holding current ($F=2.34$, $p=0.09$), or nerve activity ($F=2.17$, $p=0.11$) were observed (Fig. 11). Outward current slightly changed ($F=13.7$, $p<0.01$) due to a decrease during apamin perfusion by $11.4\pm6.1\%$ (mean decrease 31.9 ± 10.4 pA, $p<0.01$) but did not recover in the immediate washout period ($p=0.24$).

Charybdotoxin (10nM) was without significant effect on membrane resistance ($F=1.67$, $p=0.20$, $n=11$ runs on 11 cells) or nerve activity ($F=0.63$, $p=0.60$) (Fig. 12). Holding current did not change during charybdotoxin superfusion ($p=0.33$) but decreased slightly during the recovery period (mean decrease 11.4 ± 5.2 pA, $p<0.05$). Charybdotoxin decreased outward current by $12.1\pm3.0\%$ (mean decrease 47.3 ± 13.4 pA, $p<0.01$) and this did not reverse during the washout period ($p=0.10$).

DISCUSSION

The major finding of this study is that changes in glomus cell membrane K^+ conductance are poorly correlated with changes in nerve activity during brief periods of either hypoxia or application of K^+ blockers. This suggests that hypoxia-modulated inhibition of glomus cell K^+ current is not the primary mediator of the carotid body's response to hypoxia.

Passive properties

This is the first report of "perforated-patch" recordings of glomus cells in the intact carotid body. The perforated-patch method was first developed by Lindau and Fernandez, who used ATP to permeabilize a patch of mast cell membrane drawn into the patch pipette. The permeabilizing activity of ATP reduced access resistance sufficiently that, in contrast to standard whole-cell patch recordings, whole-cell currents could be recorded through the patch without dialyzing the cytoplasm (Lindau and Fernandez 1986). Other groups improved on the technique by substituting for ATP the polyene antibiotics nystatin (Horn and Marty 1988) and amphotericin (Rae et al. 1991), which are more general in their permeabilizing activities and achieve greater reductions in access resistance. Stea and Nurse recently found that recordings in isolated, cultured glomus cells

using the perforated-patch technique were more stable than with conventional patch methods (Stea and Nurse 1991).

Significantly, the cell characteristics measured here were generally consistent with those observed in preparations of dispersed glomus cells. The average R_o measured with perforated-patch access in the presence of a bicarbonate buffer was 820 M Ω , somewhat lower than what we found using conventional whole-cell recordings (1.4 G Ω). However, both sets of measurements were in the range reported for perforated-patch recordings of isolated, cultured rat glomus cells, which extends from 400 M Ω (Stea and Nurse 1991) to 2-3 G Ω (Buckler and Vaughan-Jones 1994b)

Similarly, the average glomus cell capacitance of 7.4 pF, calculated from the current transient observed with a step hyperpolarization, is also consistent with those measured in preparations of isolated cells. Comparable values have been obtained in cultured rat glomus cells (8.5 pF) (Stea and Nurse 1991) and acutely dissociated rat glomus cells (5.1 pF) (Donnelly 1993b). In slightly less than half of the cells, the transient was well fit by a single exponential with >95% of the total current in the first exponential term. In the remainder of the sample, a greater proportion of the total current was in higher-order terms, possibly suggesting gap junction communications (Monti-Bloch et al. 1993) or attachment of the patch pipette to a process rather than to the soma. Another study in this laboratory,

in which the vital dye Lucifer Yellow was used to stain recorded cells, demonstrated the presence of multiple glomus cell processes but no multi-somal staining (Donnelly 1995). This suggests that the multi-exponential current transients we observed were most likely due to sealing of the patch pipette onto a process.

Active properties

In both perforated-patch and conventional whole-cell recordings, depolarization evoked only a voltage-dependent outward current with no evidence of significant fast inward currents. Fast inward Na^+ currents have been observed in rabbit glomus cells (Peers and Buckler 1995) as well as in cultured glomus cells from neonatal rats (Stea and Nurse 1991). However, studies of mature rat glomus cells exhibit a lack of fast Na^+ currents that is consistent with our results (Fieber and McCleskey 1993).

The outward current was found to be sensitive to verapamil, TEA, and charybdotoxin; minor sensitivity to apamin was also observed. These results are consistent with prior experiments showing that in dispersed glomus cells, much of the outward current in rat glomus cells is carried by calcium-dependent K^+ channels and is inhibited by verapamil and charybdotoxin (Peers 1990a). Our finding of sensitivity of the potassium current to apamin does differ from prior observations that the outward current is

insensitive to apamin (Peers 1990a). However, the observed sensitivity is minimal even at high concentrations of apamin (100 nM). Still, it is perhaps worth noting that a component of voltage-activated outward current has been reported to be sensitive to apamin in rabbit glomus cells (Duchen et al. 1988).

Of some surprise is the observation that TEA caused a positive shift in holding current in cells recorded in the perforated-patch mode (Fig. 9). Our expectation was that at a voltage clamp of -60 mV, the membrane voltage would be positive to the Nernst potential for potassium, and net K^+ efflux would occur from the cell. Antagonism of this current with TEA would therefore be expected to result in a decrease in outward current, i.e., a net increase in the magnitude of negative holding current. Since the opposite occurred, either the Nernst potential for K^+ is positive to -60 mV or TEA was inhibiting more than just K^+ current. The former seems unlikely, since it would imply an unusually low intracellular concentration of potassium (on the order of 40 mM) to satisfy the Nernst equation. Yet this possibility cannot be entirely ruled out, since it would be consistent with our observation that the holding current does not exhibit this behavior with the conventional whole-cell patch, where the electrolyte contents of the cell might equilibrate with the potassium-rich pipette fluid to produce a more negative (and possibly non-physiologic) potassium

Nernst potential.

On the other hand, it is possible that TEA also inhibits other glomus cell membrane currents, such as chloride. TEA has been shown to inhibit chloride current in some neurons (Sanchez and Blatz 1992). If chloride were to mediate the TEA effect on holding current, the Nernst potential for chloride must be positive to -60 mV, which would require an intracellular chloride > 14 mM. This explanation would be consistent with our failure to measure the TEA effect on holding current effect in conventional whole-cell mode, where the chloride-free pipette fluid is expected to rapidly dialyze the cell interior.

Further explanation, however, would be required to explain why TEA caused a large increase in R_o in the perforated-patch mode but not with the conventional patch. One may conjecture that an intracellular factor which helps to maintain a higher membrane conductance is dialyzed from the cell in the conventional patch mode. The resulting limited membrane conduction would then exhibit a smaller relative change in response to externally-applied channel-blockers. Further investigation, however, would be required to assess this hypothesis.

The carotid body and hypoxia transduction

The experiments described address two predictions which follow from the membrane-current modulation model of carotid

body hypoxia transduction (Gonzalez et al. 1992): 1) hypoxia-induced K^+ current inhibition should occur before the nerve response to hypoxia, and 2) K^+ channel blockers should emulate severe hypoxia. Neither prediction was supported by our results.

With regard to the first prediction, during brief hypoxia, a large increase in nerve activity was always seen. Notably, however, no significant change in either holding current or R_o was seen, in contrast to the increase in magnitude of negative holding current expected if oxygen-sensitive potassium channels had been open at our baseline voltage clamp at -60 mV.

Baseline noise in our experimental precluded the measurements of very small changes (<10 pA) in holding current in response to hypoxia. Buckler and Vaughan-Jones have reported that in dispersed neonatal rat glomus cells, anoxia and hypercapnia cause a very small depolarizing current on the order of 2-7 pA (Buckler and Vaughan-Jones 1994b; Buckler and Vaughan-Jones 1994a). Such a holding current change would have been below the detection range of our recordings.

Significantly, however, as indicated by the I-V relationship shown in Figure 4, depolarization evokes a large voltage-dependent outward current. Prior studies have examined the effects of hypoxia on glomus cell membrane currents at depolarized potentials. These studies

demonstrated that outward current can be reversibly suppressed by hypoxia in depolarized isolated glomus cells. (Peers 1990b; Peers 1990c; Stea and Nurse 1991). Using these studies as a basis for comparison, we examined the effect of hypoxia on the magnitude of outward current in depolarized glomus cells in the intact carotid body. As with holding current, no significant change in outward current was observed with perforated-patch recordings. In conventional whole-cell recordings, outward current decreased during hypoxia but failed to recover in the posthypoxia period, despite a return of nerve activity to baseline spiking levels.

The lack of significant glomus cell outward current inhibition by hypoxia in the intact organ thus seems to contradict the earlier studies in isolated glomus cells. This difference might at first seem attributable to our sampling of glomus cells in the intact organ. Along these lines, three possible explanations must be considered: 1) our recordings were contaminated with nonglomus cell types, 2) only a subpopulation of glomus cells are participating in the hypoxia response, and 3) the effect of hypoxia on outward current is suppressed in the intact organ to a low level that remains physiologically significant, but undetectable by our methods.

The suggestion that nonglomoid cells contaminated our recordings is unlikely, since glomus cells constitute 80% of

the carotid body volume and should therefore dominate any random sampling of cells (McDonald and Mitchell 1975). In addition, the presence of a voltage-activated outward current distinguishes glomus cells from type II or sustentacular cells, which have been reported to lack voltage-gated outward current (Duchen et al. 1988; Urena et al. 1989; Donnelly 1993b). Our identification of glomus cells is further supported by the observation that the outward current is rapidly inhibited by the addition of the calcium-channel blocker verapamil to the perfusate, an effect seen previously in isolated rat glomus cells (Peers 1990b).

The issue of whether the nerve response to hypoxia is modulated by only a subpopulation of glomus cells is less certain. We did observe some heterogeneity in the response of outward current to hypoxia, as demonstrated in the contrasting current tracings in Figures 6 and 7. Furthermore, McDonald and Mitchell tentatively subdivided populations of glomus cells on the basis of microanatomical features such as the size of dense cored granules and synapses to nerve endings (McDonald and Mitchell 1975). Significantly, however, no electrophysiological criteria by which subpopulations of isolated glomus cells might be distinguished have ever been reported, despite numerous studies of such cells. It remains possible, of course, that the spatial distribution and interrelationships of glomus

cells within the carotid body leads to physiologically distinct subpopulations of cells in the intact organ. For instance, a significant oxygen gradient may exist between glomus cells in the periphery of the organ and cells that are centrally situated. The inability of our current optical apparatus to resolve the precise spatial locations of the cells we recorded within the carotid body prevents us from addressing this important question.

The third possibility is that in the intact organ, cell-cell interactions may suppress the membrane current response to hypoxia to a level that remains physiologically significant, but is undetectable to our apparatus. As noted above, the 2-7 pA depolarizing current measured by Buckler and Vaughan-Jones at resting voltages (Buckler and Vaughan-Jones 1994b; Buckler and Vaughan-Jones 1994a) would have been below the threshold of the sensitivity of our recording apparatus, assuming such a current is not enhanced by depolarization.

Such a small depolarizing current might plausibly be significant if it served to modulate spontaneous action potentials. For instance, rabbit glomus cells exhibit a fast voltage-dependent sodium current (Lopez-Barneo et al. 1993) which allows for the generation of trains of action potentials. The rate of spontaneous spiking in these cells might conceivably be affected by small resting potassium currents which are modulated by hypoxia.

Rat glomus cells do not exhibit such spontaneous spiking activity, however (Donnelly 1993b), and any change in outward currents at rest therefore can serve only to modulate the membrane potential. Therefore, if small depolarizing currents are responsible for mediating the hypoxia response, they must be able to cause a change in membrane potential large enough to activate a voltage-gated signalling mechanism, for instance an opening of calcium channels. Given the average glomus cell membrane resistance of about 800 M Ω (as measured in our preparations with perforated-patch access), a decrease of 5 pA in outward current would be expected to depolarize the glomus cell membrane by about 4 mV. The resting potential of our glomus cells was not measured in these experiments, but has been characterized by Buckler and Vaughan Jones as being in the range of -40 to -60 mV (Buckler and Vaughan-Jones 1994b). A membrane depolarization of 4 mV from this range of resting potentials would not be expected to cause an increase in calcium influx, given the I-V relationships of glomus cell calcium channels published previously (Fieber and McCleskey 1993). It is, of course, possible that the resting potential of the glomus cells in the intact carotid body is positive to -40 mV, i.e., in the voltage range where small changes in membrane voltage may activate voltage-gated calcium and sodium currents. However, in that case, one would expect hypoxia transduction to be extremely sensitive

to potassium conductance.

One way to examine the possibilities either that subpopulations of glomus cells mediate the hypoxia response, or that hypoxia transduction is highly sensitive to potassium conductance, is to test the effects of potassium channel blockers on the nerve response of the rat carotid body. As noted above, a second prediction of the membrane current model of hypoxia transduction is that potassium channel blockers added to the perfusate of the intact organ should emulate severe hypoxia. This prediction should remain valid even if only a subpopulation of cells responded to hypoxia through a depolarizing inhibition of potassium current. Moreover, a mechanism of hypoxia transduction highly sensitive to potassium conductance would be expected to be highly sensitive to potassium channel blockers.

With regard to this prediction, however, potassium channel blockers applied to the intact carotid body did not evoke a significant increase in nerve activity. TEA is a quaternary ammonium compound with a diameter of about 8 Å, approximating that of a hydrated potassium ion (Aidley 1989). Many varieties of voltage-dependent potassium channels are blocked by TEA, though the half-blocking concentration ranges over several orders of magnitude, from 0.2 mM to greater than 100 mM (Hille 1992; Pongs 1992). In our carotid body preparation, despite significant (>50%) outward current inhibition by 20 mM TEA, only slight changes

in nerve activity were observed. Nerve activity was similarly unresponsive to the potassium channel toxins charybdotoxin and apamin, which inhibited outward current to a lesser degree. Furthermore, the sinus nerve remained responsive to hypoxia in the presence of TEA, demonstrating that TEA did not confound measurements of nerve activity through a direct suppressive effect on the nerve.

Significantly, results of a more recent study in dispersed glomus cells are consistent with our data regarding the effects of potassium channel blockers on glomus cells. Buckler has reported that externally applied charybdotoxin, TEA, and 4-AP have no effect on glomus cell intracellular calcium concentration as measured by fluorescence of the calcium indicator probe indo-1 (Buckler 1995). In contrast, hypoxia evokes a large rise in $[Ca^{2+}]_i$, suggesting that modulation of potassium channels sensitive to these blockers is not required for causing calcium-dependent secretion of neurotransmitters in glomus cells.

A difficulty in interpreting our data in the whole organ is determining the degree to which the applied potassium channel blockers are able to penetrate the intact carotid body. In particular, if only a subpopulation of glomus cells is mediating the nerve response to hypoxia, and that subpopulation is sequestered from toxins in the perfusate by a central location within the organ, we might not expect to have been able to evoke a nerve response with

K⁺ channel blockers.

This issue remains an open question which is not addressed in the present study. However, it appears unlikely that the effects of the K⁺ channel blockers on nerve activity are diffusion limited, since the diffusion distance is small to begin with (rat carotid body diameter $\approx$ 400 μ m), and a recent study in this laboratory examining the effects of 4-aminopyridine on sinus nerve activity over a 15-20 minute period showed no time-dependent increase in nerve activity during that period, as might be expected for a diffusion-limited effect (Doyle and Donnelly 1994). It is also worth noting that another study in this laboratory demonstrated that in an intact carotid body preparation, sinus nerve activity increases promptly in response to externally applied neurotransmitters in the perfusate such as dopamine and acetylcholine (Kholwadhwa and Donnelly 1992). Both dopamine (m.w. 146) and acetylcholine (m.w. 154) are of similar molecular weight to TEA (m.w. 130), and all are singly positively charged at physiologic pH. Acetylcholine, in particular, shares with TEA a quaternary nitrogen moiety. It might be inferred from these data that TEA at least is probably able to diffuse to active junctions between the sinus nerve and glomus cells where the hypoxia response is being transduced.

In contrast, the molecular weights of the polypeptide toxins apamin and charybdotoxin are on the order of several

thousand Daltons (Strong 1990), and thus their diffusion properties within the carotid body cannot be inferred from above data. However, a recent abstract does report the results of charybdotoxin experiments with a slightly different focus, examining whether charybdotoxin modulates the nerve response to hypoxia in the intact carotid body (Pepper et al. 1995). Interestingly, the experiments showed that at levels of PO_2 of 100 and 75 mmHg, charybdotoxin caused a significant increase in nerve activity, but not at levels of PO_2 in the range of 150-400 mmHg. These results do not conflict with ours, since we measured the effects of charybdotoxin only at a PO_2 of 150 mmHg, and moreover the concentration of charybdotoxin used by Pepper and colleagues is tenfold higher than the concentration we used. The interpretation of these observations is unclear; if inhibition of potassium channels is the primary step in the nerve response to hypoxia, then potassium channel blockers should be able to emulate severe hypoxia regardless of the perfusate PO_2 . On the other hand, it remains possible that potassium channels play a secondary role in modulating hypoxia chemotransduction. In any event, these new experiments support our assumption that even the larger polypeptide channel blockers probably penetrate to the physiologically relevant glomus cells in the intact organ.

In summary, our results strongly suggest that the sinus nerve response to hypoxia in the rat carotid body is not

primarily mediated by inhibition of a glomus cell K^+ current. In the intact organ, we found that hypoxia does not inhibit glomus cell outward current in most cell recordings, in contrast to prior studies in isolated glomus cells. Furthermore, the effects of external K^+ blockers failed to emulate severe hypoxia. These results call into question the most widely-held model of carotid body hypoxia chemotransduction, centered around the inhibition of a O_2 -sensitive potassium channel (Gonzalez et al. 1992). They imply that hypoxia transduction is more complicated than previously postulated, possibly involving intracellular messengers which directly evoke secretion.

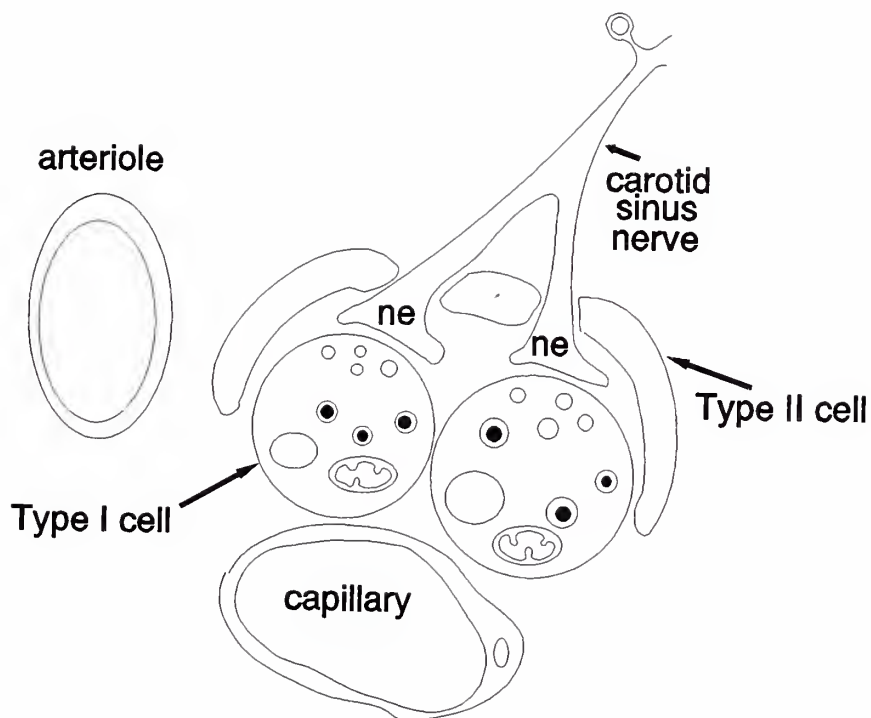


Figure 1. Simplified schematic of the cellular arrangement of the carotid body. Key: ne, sinus nerve endings; Type I and Type II cells refer to glomus and sustentacular cells, respectively. Sympathetic innervation is not shown. Figure adapted from Nye (Nye 1994).

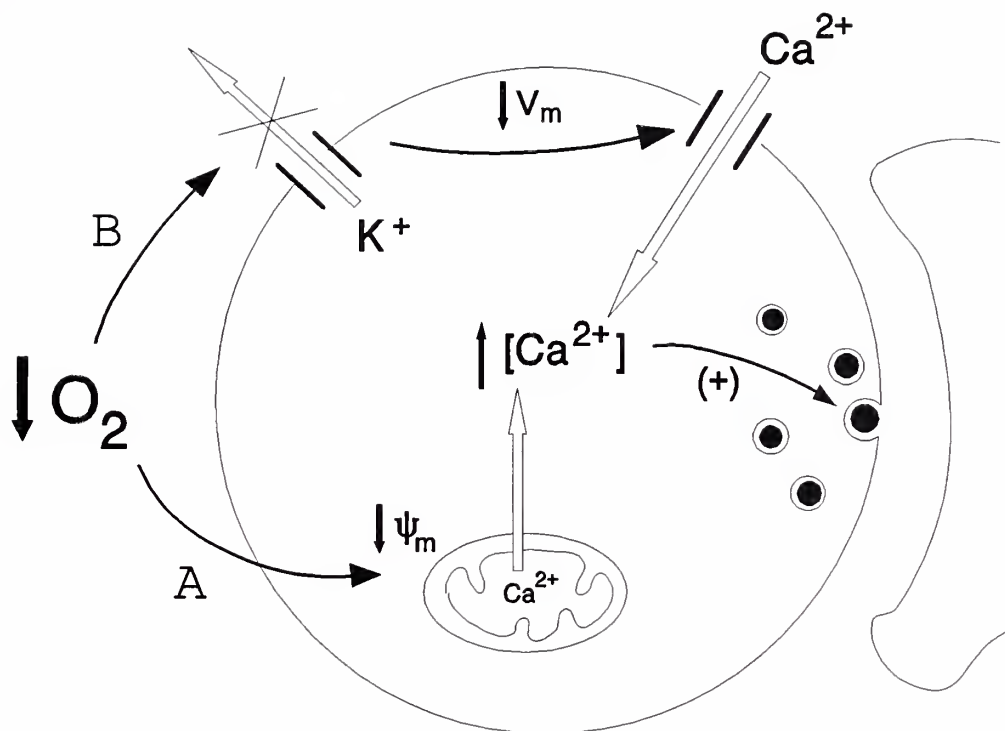


Figure 2. Two models of hypoxia chemotransduction in the carotid body glomus cell. (A) Metabolic model proposed by Biscoe and Duchen: hypoxia leads to mitochondrial depolarization (decrease in ψ_m), causing release of calcium from mitochondrial stores. (B) Plasma membrane model proposed by Lopez-Barneo and coworkers: hypoxia leads to closure of oxygen-sensitive K^+ channels, resulting in cell membrane depolarization (decrease in V_m) and calcium influx through voltage-dependent calcium channels. Note that in both models, hypoxia leads to an increase in intracellular $[Ca^{2+}]$, which causes neurotransmitter secretion and stimulation of sinus nerve firing. The plasma membrane model is currently more widely-held, and is the model whose predictions are directly addressed by our experiments.

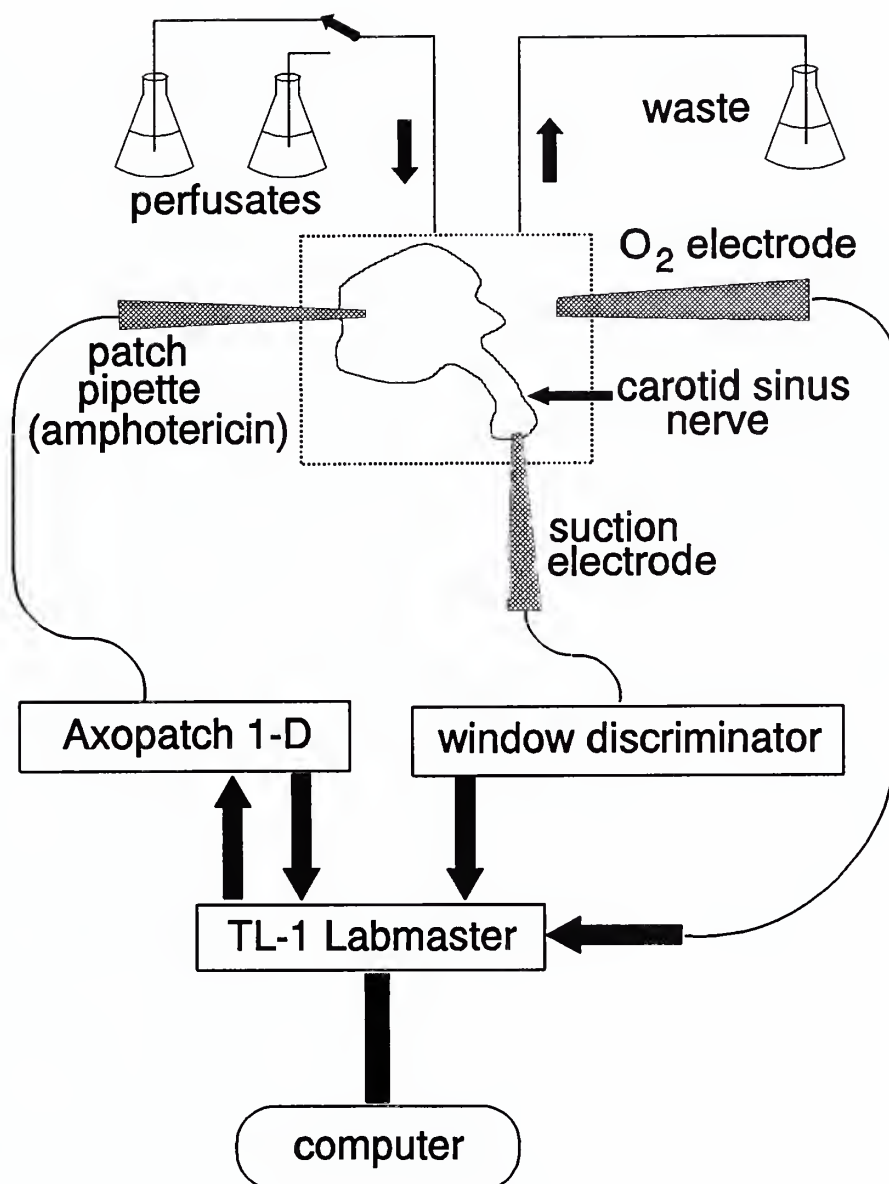


Figure 3. Schematic of the experimental apparatus. The intact carotid body is shown at the center of the perfusion chamber (dotted box). The patch pipette is sealed onto a glomus cell located in the main body of the organ. A suction electrode records sinus nerve activity, and an O₂ electrode measures the perfusate PO₂. The system is thus designed for simultaneous measurements of glomus cell membrane current, nerve activity, and PO₂ in the intact carotid body. The perfusate is varied for the different experimental runs to bathe the organ in either a hypoxic environment or a solution containing potassium channel toxins.

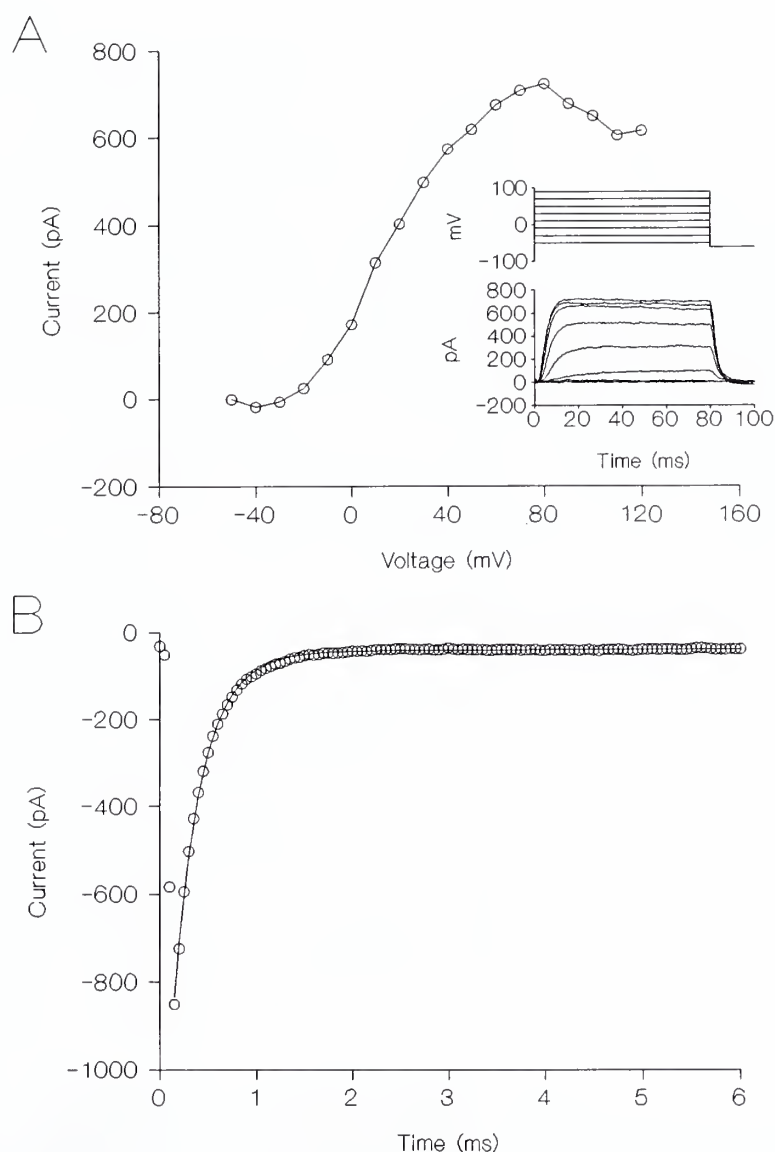


Figure 4. Cell I/V characteristics are consistent with previous recordings of isolated glomus cells. (A) Current-voltage relationship of an intact glomus cell under normoxic conditions. Shown are leak-subtracted, peak whole-cell outward currents evoked by successive depolarizations from -60 mV (holding potential) over the range of -50 mV to $+80$ mV. *Inset:* Original oscillographic tracings of leak-subtracted current. (B) Current relaxation following a step hyperpolarization is consistent with a single RC circuit. Current data points (open circles) following a step hyperpolarization from -60 mV to -80 mV are fit to a 3rd order exponential: $I = 0.277 \text{ ms} @ -770 \text{ pA} + 1.1 \text{ ms} @ -29 \text{ pA} + 3.5 \text{ ms} @ -1.1 \text{ pA}$. Calculated values for $R(\text{access}) = 23 \text{ Mohm}$; $R_o = 2.18 \text{ Gohm}$; $C_m = 11.7 \text{ pF}$.

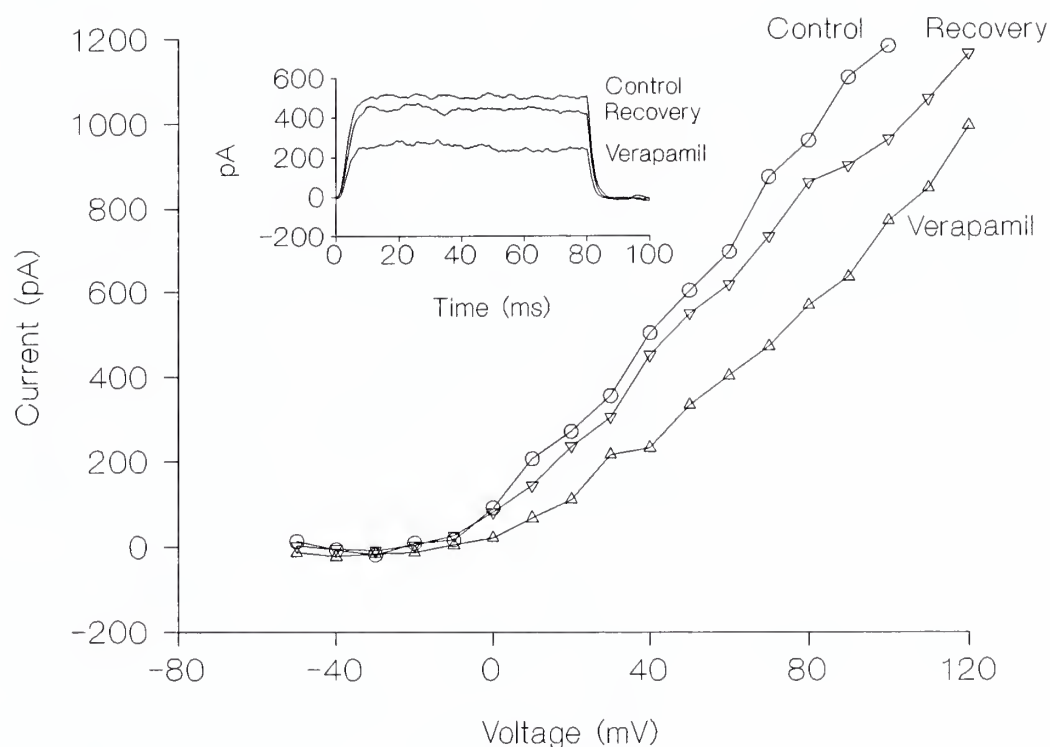


Figure 5. Verapamil inhibits the outward current of purported glomus cells, consistent with previous studies of isolated glomus cells (Peers 1990c). Current-voltage relationship for a glomus cell recorded using the perforated-patch technique, before, during and following perfusion with a calcium channel blocker, verapamil ($10\mu\text{M}$). Current values are peak, leak-subtracted currents during a depolarization step over a range of -45 to $+120\text{mV}$ from a holding potential of -60mV . *Inset:* Oscillographic tracings of leak subtracted current during voltage steps to $+40\text{mV}$, before, during a following exposure to verapamil.

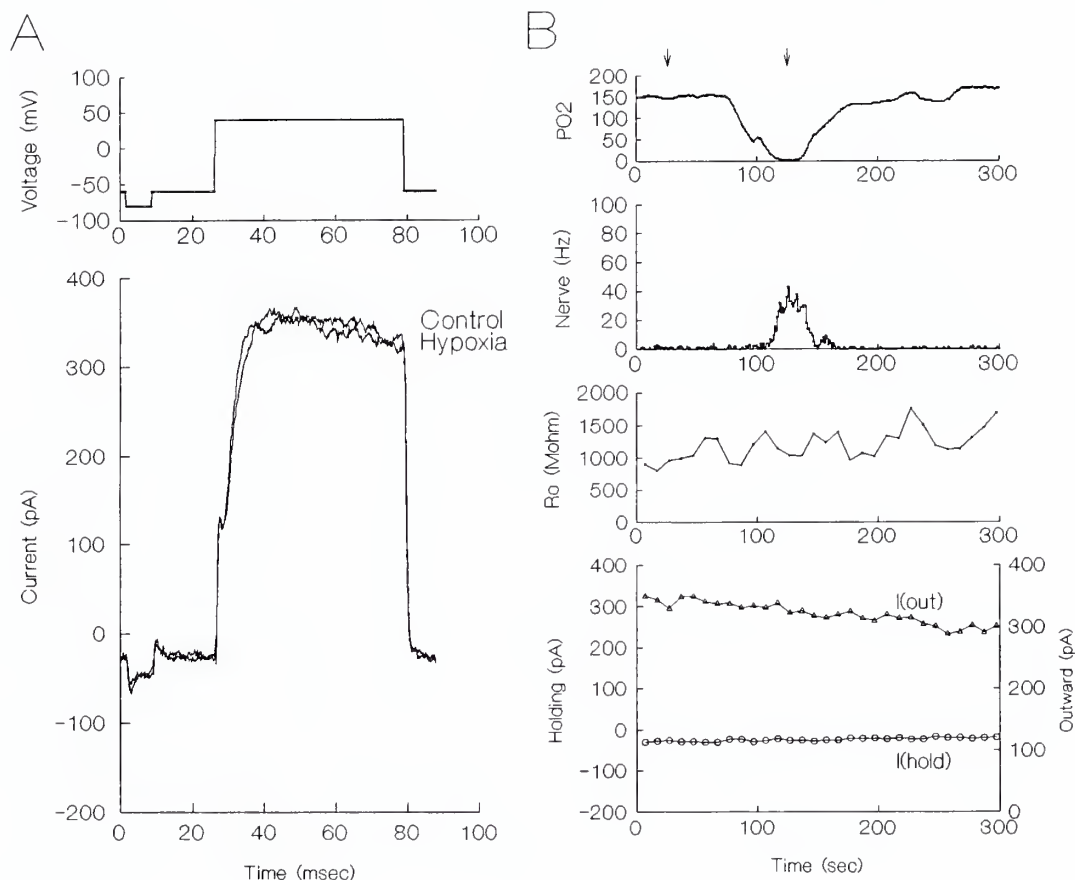


Figure 6. Recordings of a glomus cell which failed to respond to brief period of hypoxia. (A) Oscillographic current tracing of a cell recorded using perforated-patch access during a step hyperpolarization from -60 to -80 mV and from a step depolarization from -60 mV to $+40$ mV. (B) Time dependent changes in nerve and cell characteristics. *Top*, Perfusate PO₂ measured close to the organ. Arrows indicate the time points of the oscillographic tracings shown in A. *2nd*, pauci-fiber sinus nerve activity. *3rd*, calculated input resistance for the recorded cell. *4th*, changes in outward current and holding current.

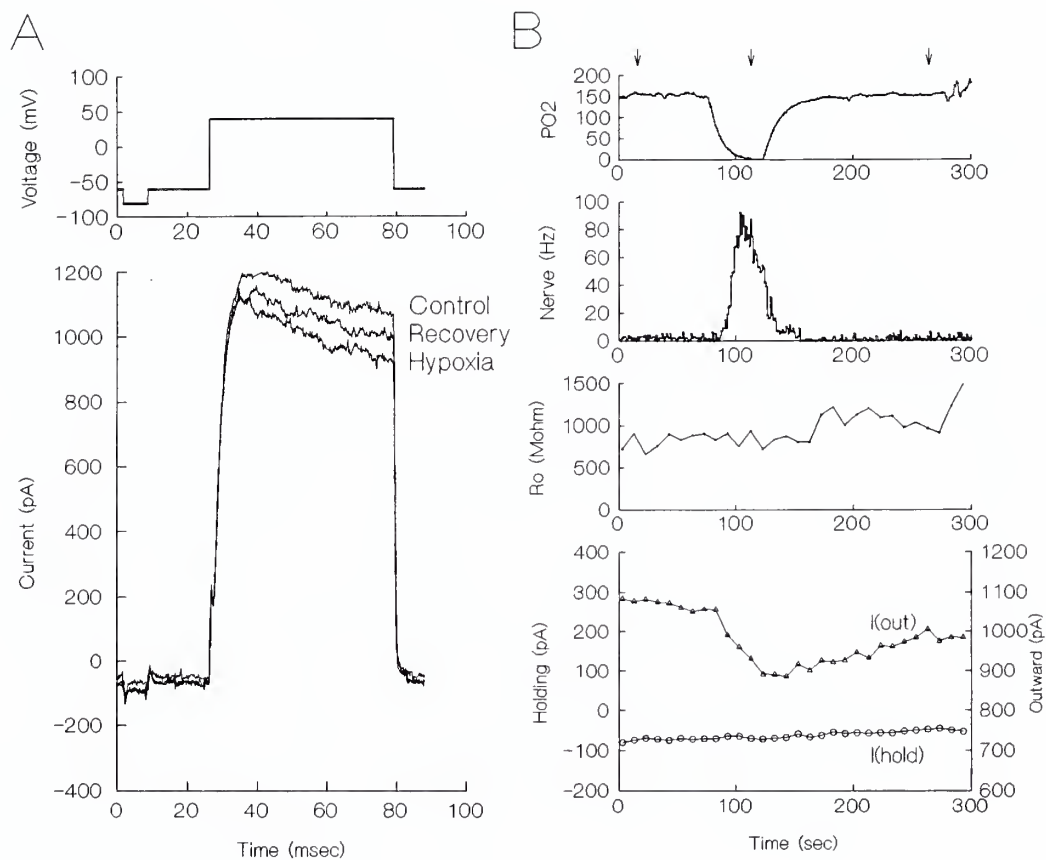


Figure 7. Recordings of a glomus cell in which hypoxia inhibited outward current. Cell was recorded using perforated-patch access. Figure legend as in figure 6.

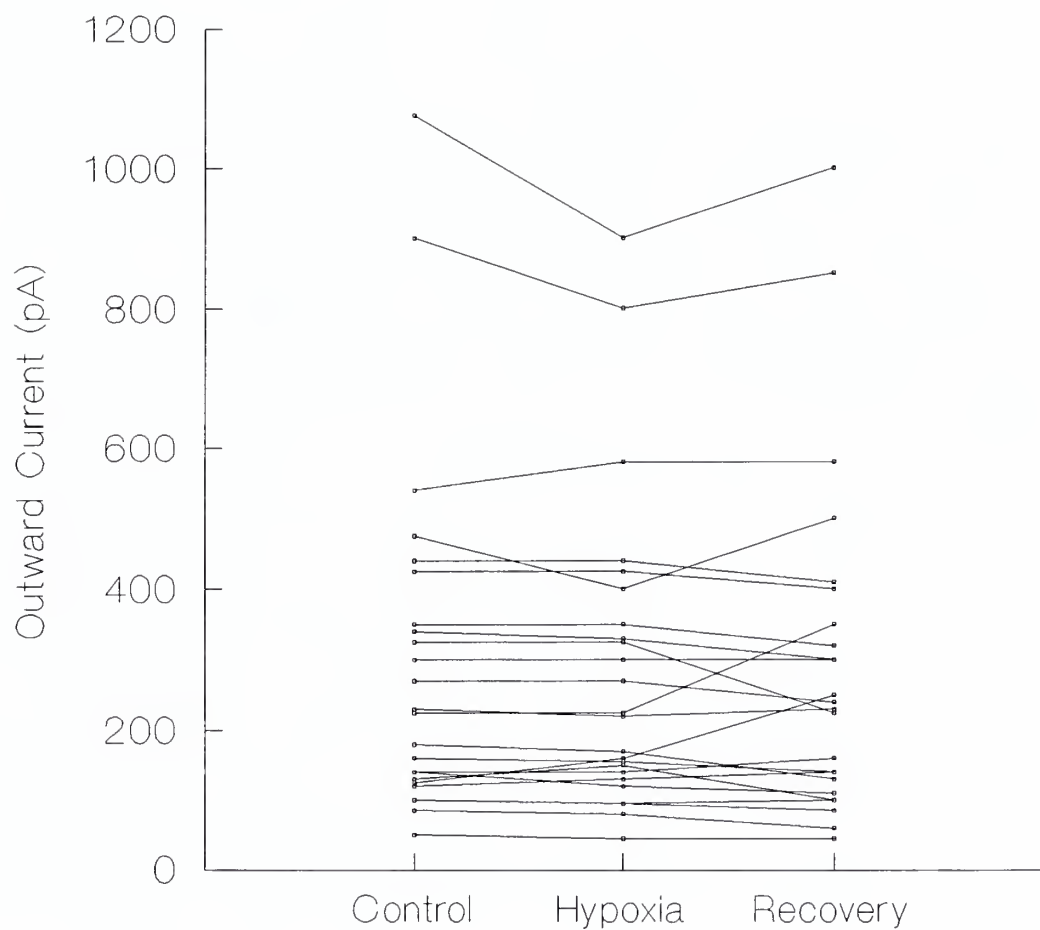


Figure 8. Hypoxia failed to alter outward current in most glomus cells. Distribution of changes in outward current measured at $+40\text{mV}$ of 24 cells recorded using perforated-patch access, before, during and following brief periods of hypoxia.

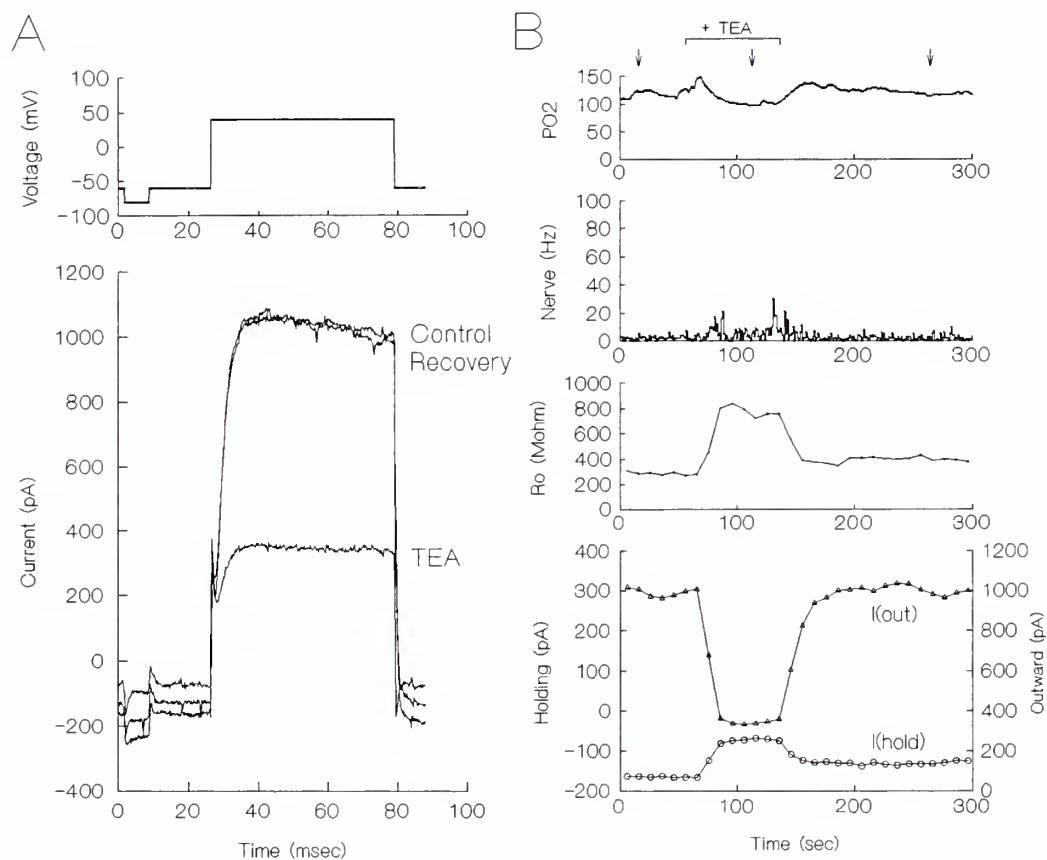


Figure 9. K^+ channel blockade with TEA (20mM) failed to inhibit outward current but caused little change in nerve activity. Cell recorded using perforated-patch access. Legends are the same as in figure 6. Note the large inhibition of cell outward current but little change in nerve activity.

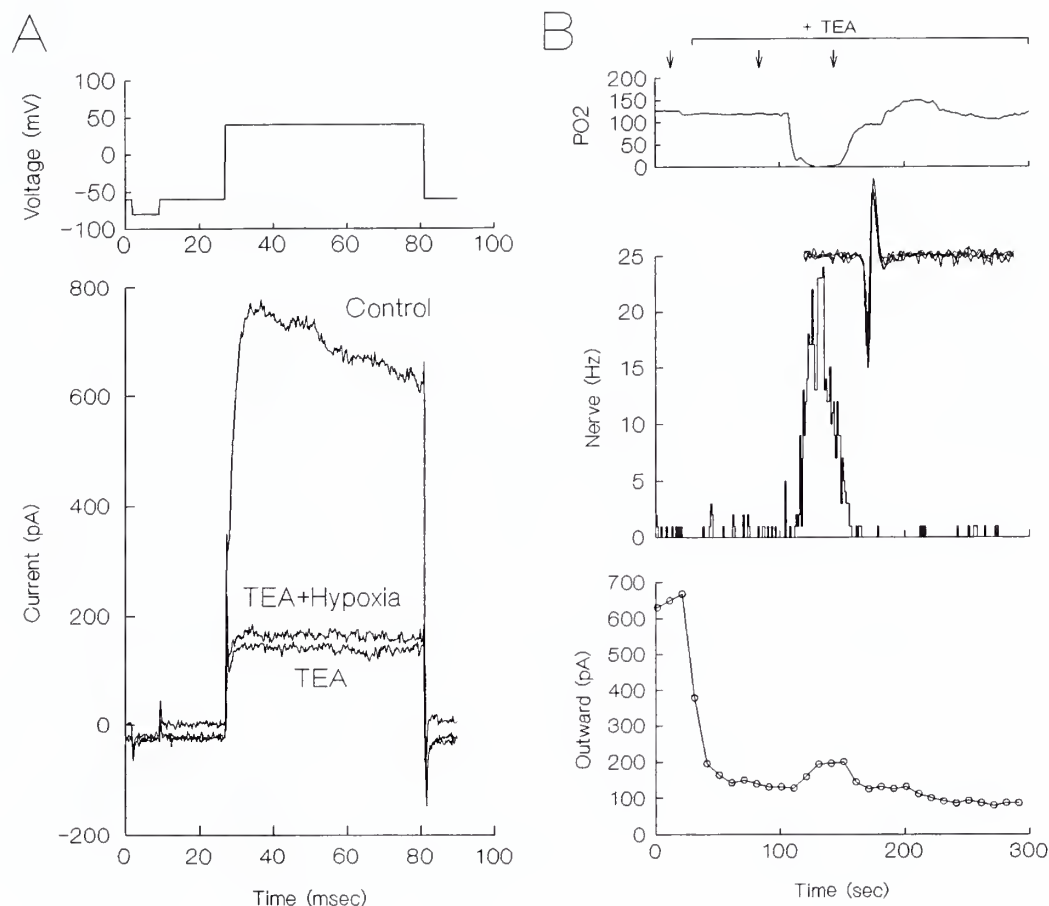


Figure 10. K^+ channel blockade with TEA (20mM) does not ablate the nerve response to hypoxia. Cell was recorded in conventional whole-cell recording mode, and single-fiber activity was discriminated from the nerve recording. (A) Oscillographic current tracing recorded prior to, during TEA exposure and during the peak nerve response to anoxia in the presence of TEA. (B) *Top*, Perfusate PO_2 measured close to the organ. Arrows indicate the time points of the oscillographic tracings shown in A. *Middle*, single-fiber nerve recording. *Inset*, superimposed tracings of the extracellularly recorded nerve action potential. *Lower*, changes in outward current.

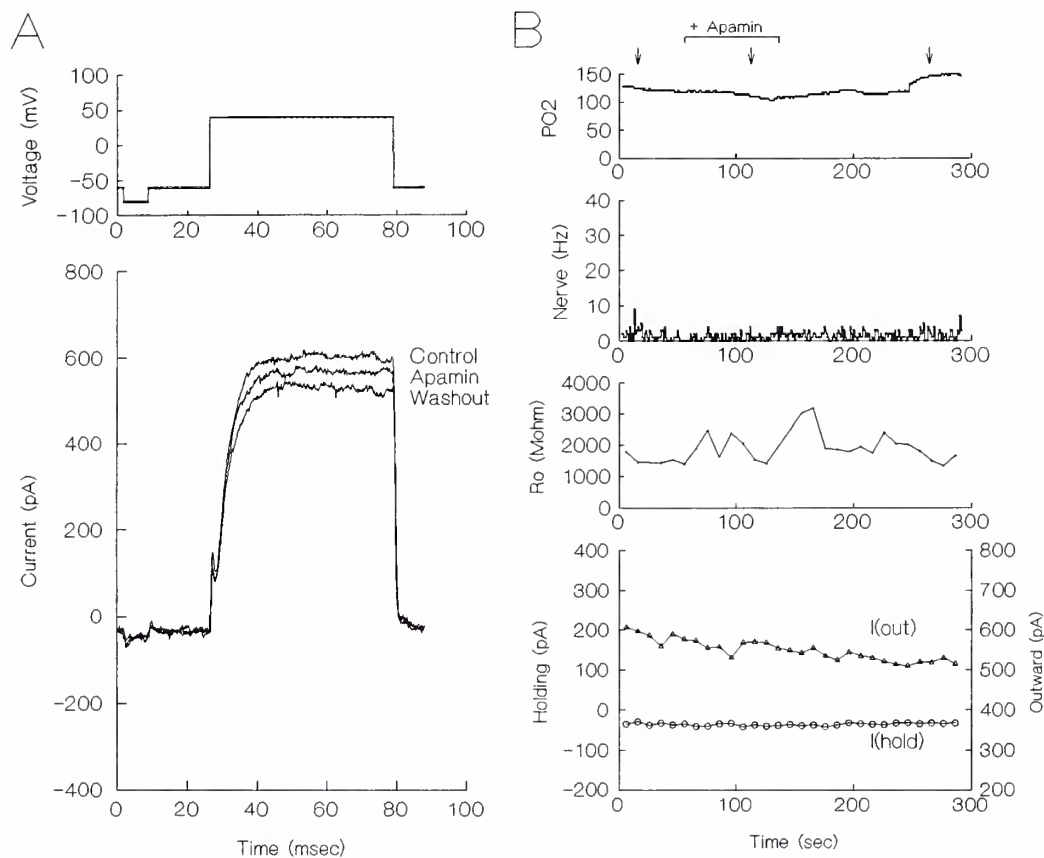


Figure 11. Apamin (100nM) causes little inhibition of outward current and no change in nerve activity. Cell was recorded using the conventional whole-cell technique. Legends are the same as in figure 6.

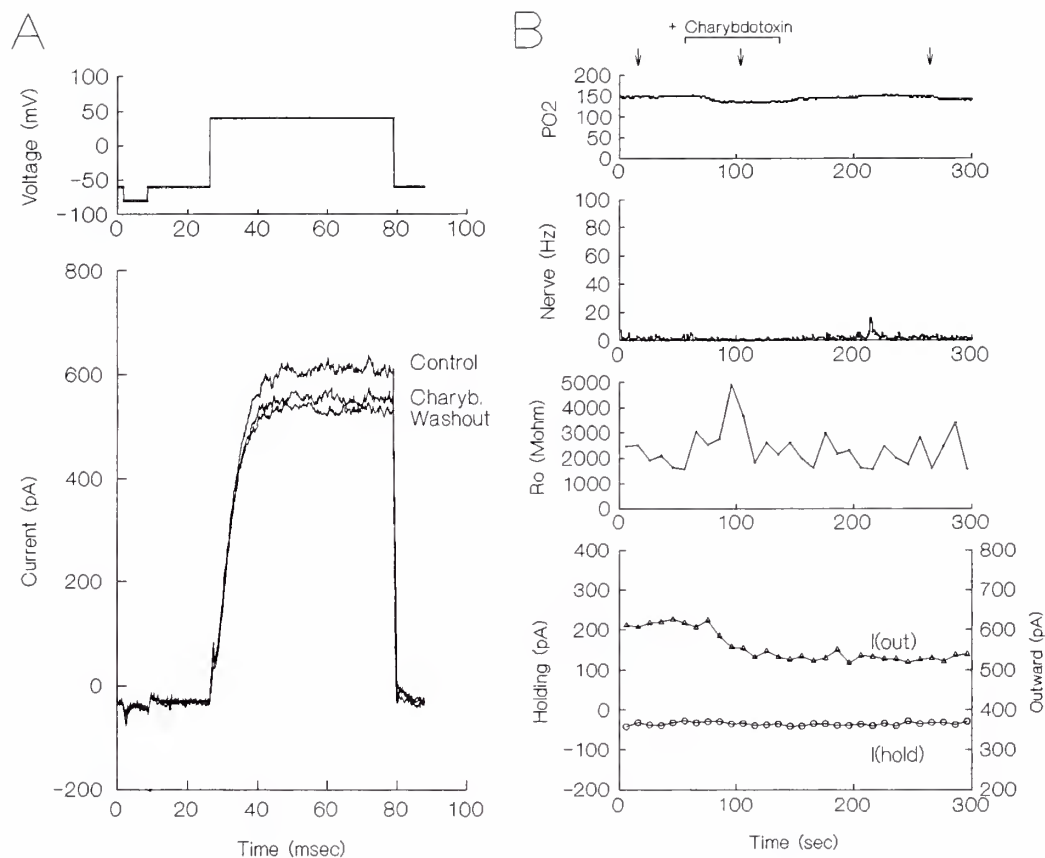


Figure 12. Charybdotoxin (100nM) inhibits outward current but causes no change in nerve activity. Cell was recorded using the conventional whole-cell technique. Legends are the same as in figure 6.

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